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Paolo Jorio
Roberto Prato
Luigi Boggio
Rinaldo Plebani
Corrado Modugno
Simone Bongiovanni
Luigi Franzolin
Elena Cerbaro
M. Cristina Baldini
Giancarlo Revelli
Mauro Eccetto
Raffaele Borrelli

Fabio D'Angelo
Mirko Bergadano
Matteo Bellemo
Matteo Maccagnan
Daniele Cernuzzi
Stefano Manconi Dirrigl
Raffaella Barbuto

Robert Scotti (US IP Att.)
Satsuki Takasaka
Francesco Fiussello
Giovanni Lo Cigno

Andrea Bernotti
Paolo Lovino
Cesare Bosman
Stefania Berta
Bruno Zamprogno
Simone Mangini

Edoardo Mola
Lorenzo Nannucci
Manuela Giannini
Cristina Rolando
Andrea Fiorini
Michele Di Sciuva
Lidia Casciano
Alessandro Putzolu
Erika Andreotti
Gerardo Valeriano

Legal Advisers:
Claudio Costa
Maria Teresa Saguatti
Chiara Luzzato
Alberto Improda
Raffaella Arista

STUDIO TORTA

JORIO, PRATO, BOGGIO & Partners
Via Viotti, 9 - 10121 Torino - Italy
Tel. +39-011.561.13.20; Fax +39-011.562.21.02
Videoconference +39-011.518.42.48 (6 ISDN channels H.320)
E-mail: info@studiotorta.it Internet: www.studiotorta.com
Industrial Property Consultants - Established in 1879
European and Italian Patent Attorneys
European and Italian Trade Mark and Design Attorneys
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Dear Sirs,

RE: International Application No. PCT/IT2006/000049 filed on January 27, 2006
in the name of **ALMA MATER STUDIORUM – UNIVERSITA' DI BOLOGNA**

With reference to the application in object, we enclose herewith the priority document of the basic US Patent application.

Yours faithfully,

Dr. Ing. Elena CERBARO

PA 1425224

THE UNITED STATES OF AMERICA

TO ALL TO WHOM THESE PRESENTS SHALL COME:

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February 09, 2006

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APPLICATION NUMBER: *60/647,498*
FILING DATE: *January 27, 2005*

THE COUNTRY CODE AND NUMBER OF YOUR PRIORITY APPLICATION, TO BE USED FOR FILING ABROAD UNDER THE PARIS CONVENTION, IS *US60/647,498*

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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. EV333073539US

INVENTOR(S)		
Given Name (first and middle [if any])	Family Name or Surname	Residence (City and either State or Foreign Country)
Michela	ROSINI	Bologna, Italy

Additional inventors are being named on the 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max):

MULTIPOTENT ANTI-ALZHEIMER DRUGS

CORRESPONDENCE ADDRESS

Direct all correspondence to:

☐ The address corresponding to Customer Number:

OR

☑ Firm or Individual Name: Law Offices of Albert Wai-Kit Chan, LLC

Address: World Plaza, Suite 604
141-07 20th Avenue

City: Whitestone | State: New York | Zip: 11357

Country: U.S.A. | Telephone: (718)-357-8836 | Fax: (718)-357-8615

ENCLOSED APPLICATION PARTS *(check all that apply)*

☐ Application Data Sheet. See 37 CFR 1.76

☑ Specification *Number of Pages* 19

☑ Drawing(s) *Number of Sheets* 4

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Application Size Fee: If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

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TOTAL FEE AMOUNT ($): 200.00

☐ Applicant claims small entity status. See 37 CFR 1.27.

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SIGNATURE Albert Wai Kit Chan

Date 1/27/05

TYPED or PRINTED NAME Albert Wai-Kit Chan

REGISTRATION NO. 36,479 *(if appropriate)*

TELEPHONE (718) 357-8836

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First Named Inventor	Michela ROSINI	Docket Number	1033-PRO

INVENTOR(S)/APPLICANT(S)

Given Name (first and middle [if any])	Family or Surname	Residence (City and either State or Foreign Country)
Vincenza	ANDRISANO	Bologna, Italy
Manuela	BARTOLINI	Bologna, Italy
Carlo	MELCHIORRE	Bologna, Italy

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s) : ROSINI, et al.

U.S. Serial No.: Not Yet Known

Filed : January 27, 2005

For : MULTIPOTENT ANTI-ALZHEIMER DRUGS

Law Offices of Albert Wai-Kit Chan, LLC
World Plaza, Suite 604
141-07 20th Avenue
Whitestone, NY 11357

January 27, 2005

Commissioner for Patents
P.O. Box 1450
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Mail Stop Provisional Application

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Printed Name: Alex Chay Chang

Respectfully submitted,

Albert Wai-Kit Chan
Registration No. 36,479
Attorney for Applicant(s)
Law Offices of
Albert Wai-Kit Chan, LLC
World Plaza, Suite 604
141-07 20th Avenue
Whitestone, New York 11357
Tel: (718) 357-8836
Fax: (718) 357-8615
e-mail: kitchanlaw@aol.com

Dkt. #1033-PRO

Application for United States Letters Patent

To all whom it may concern:

Be it known that **Michela ROSINI, Vincenza ANDRISANO, Manuela BARTOLINI,** and **Carlo MELCHIORRE**

have invented certain new and useful improvements in

MULTIPOTENT ANTI-ALZHEIMER DRUGS

of which the following is a full, clear, and exact description.

Multipotent Anti-Alzheimer Drugs

Abstract:

The coupling of two different pharmacophores, each endowed with different biological properties, afforded the hybrid compound lipocrine (7), whose biological profile was markedly improved relative to those of prototypes tacrine and lipoic acid. Lipocrine is the first compound that inhibits the catalytic activity of AChE and AChE-induced amyloid-β aggregation and protects against reactive oxygen species. Thus, it emerged as a valuable pharmacological tool to investigate Alzheimer's disease and as a promising lead compound for new anti-Alzheimer drugs.

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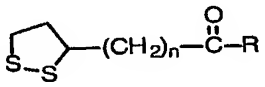
Alzheimer's disease (AD), the most common cause of dementia, is a complex neurological affection that is clinically characterized by loss of memory and progressive deficits in different cognitive domains. The consistent neuropathologic hallmark of the disorder, generally noted on postmortem brain examination, is a massive deposit of aggregated protein breakdown products, amyloid-β (Aβ) plaques and neurofibrillary tangles. Even if the primary cause of AD is still speculative, Aβ aggregates are thought to be mainly responsible for the devastating clinical effects of the disease.[1] In recent years, significant research has been devoted to the role of free radical formation, oxidative cell damage, and inflammation in the pathogenesis of AD, providing new promising targets and validated animal models.[2] To date, however, the enhancement of the central cholinergic function is the only clinically effective approach.[3,4] The intensive research of drugs that can improve the cholinergic transmission in AD has produced so far four approved acetylcholinesterase (AChE) inhibitors, that is, tacrine (TC),[5] donepezil,[6] rivastigmine,[7] and galantamine.[8] However, these drugs have been approved for the symptomatic treatment of AD because they do not address the etiology of the disease for which they are used.

It is therefore necessary to discover pharmacological instruments that are able to act as far upstream as possible in the neurodegenerative cascade and, because of the multifaceted etiology of AD, able to hit different selected targets. The aim of this communication is to provide new multipotent compounds, i.e., single molecules that can exhibit more pharmacological properties simultaneously,

such as the enhancement of the cholinergic transmission and inhibition of Aβ accumulation and oxidative stress, leading to a synergic and effective treatment of AD.

To this end, we applied a design strategy in which distinct pharmacophores of two different drugs were combined in the same structure leading to hybrid molecules. In principle, each pharmacophore of these new drugs should retain the ability to interact with its specific site(s) on the target and consequently to produce specific pharmacological responses that taken together should block or hopefully cure the neurodegenerative process leading to AD. To obtain proof of concept for this proposal, we chose TC and lipoic acid (LA) as prototype drugs to be combined in the same structure because of their well-established biological properties (Figure 1). In fact, LA is a universal antioxidant,[9-12] which was shown to protect neurons against cytotoxicity induced by Aβ[13] and to stabilize cognitive functions in patients with AD,[14] whereas TC was the first AChE inhibitor approved for AD treatment.[5] AChE is the enzyme involved in the hydrolysis of the neurotransmitter acetylcholine (ACh) at cholinergic synapses in the central and peripheral nervous system. Inhibitors of AChE activity promote an increase in the concentration and the duration of action of synaptic ACh, thus causing an enhancement of the cholinergic transmission through activation of the synaptic nicotinic and muscarinic receptors. However, achievement of potent inhibitors of the AChE catalytic site would not represent a significant improvement unless there is a concomitant inhibition of the peripheral anionic site (PAS) of the enzyme, which is associated with the neurotoxic cascade of AD through AChE-induced Aβ aggregation.[15,16] For this reason, the introduction onto the TC structure of a side chain, namely, an LA fragment following the two routes shown in Figure 1, should hopefully combine antioxidant properties with the ability to interact with PAS.

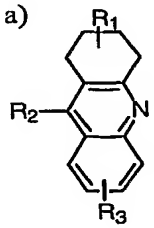
The strategy has been successfully utilized for other compounds more than tacrine. The general structure of lipoic acid derivatives can be schematized as such:



Where n = 1-4;

Examples where R =:

a)



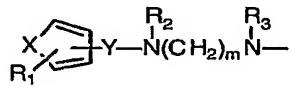
- R_1 = H, alkylamine, nitroalkyl, halogen, hydroxy
- R_2 = alkanediamine, amino
- R_3 = halogen, H, methoxy

The examples are compounds (1-8) reported in the enclosed document.

An other example is an inferior homologous of **8**:

RM11: n = 1, R_1 = 3-ylmethylamine, $R_2 = NH_2$, R_3 = 6-chloro;

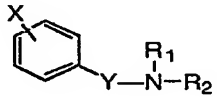
b)



Example:

MR106: n = 4; X = CH=CH, R_1 = 2-MeO, m = 6; R_2 = Et, R_3 = H.

c)



R_1 = alkyl, R_2 = alkyl, alkylamine

Y = alkyl

X = OH, O-carbamoyl, O-

An example is **MR112**: Y = MeCH, $R_1 = R_2$ = Me, X = O-.

Compounds **1-8** were synthesized by coupling tetrahydroacridine intermediates **9-16** with LA in the presence of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI), according to Scheme 1 (see Supporting Information). No attempt was made to separate the enantiomers of **1-7** because it was reported that stereochemistry is not relevant for the protective effect of LA against oxidative cell damage.[10] In addition, concerning **8**, the diastereomers were not separated because it was the weakest AChE inhibitor among the investigated compounds.

To determine the potential interest of **1-8** for the treatment of AD, their AChE inhibitory activity was determined by the method of Ellman et al.[17] on human recombinant AChE. Moreover, to study further the biological profiles of **1-8**, their butyrylcholinesterase (BChE) inhibitory activity was also assessed by the same method on BChE from human serum. To allow comparison of the results, **15**, TC, and LA were used as the reference compounds (Table 1⊖). It is evident that all compounds were effective inhibitors of AChE and BChE and significantly more potent than prototype TC with the exception of **8**. Modifying the chain length between the two nitrogen atoms of the lateral chain,

affording **1-7**, affected the affinity for AChE and BChE. Optimum inhibition of AChE was observed for **2**, having three methylene units. As expected,[18] the insertion of a chlorine atom into the acrydine system, affording **7** (lipocrine), produced an 85-, 1676-, and 28-fold increase in AChE inhibition relative to **15**, TC, and **2**, respectively. As one would expect, LA did not inhibit either enzyme. The finding that **8** was 4308-fold less potent than **2** in inhibiting AChE activity suggests that the insertion of the lipoyl fragment on the nitrogen atom at position 3 of **16** resulted in a highly negative effect on the interaction mechanism with the enzyme.

Inhibition of AChE activity by **7** was very fast and not time-dependent because 50% of enzyme inactivation produced by 0.253 nM following a 1 min incubation was not significantly different ($p > 0.01$) from the inhibition observed up to a 40 min incubation. The estimates of competitive inhibition constants K_i calculated for **7** and TC were 0.155 ± 0.046 and 0.151 ± 0.016 μM, respectively, whereas the graphical analysis of steady-state inhibition data for **7** is shown in Figure 2. An analysis of the Lineweaver-Burk reciprocal plots of **7** reveals that there are an increasing slope and an increasing intercept with higher inhibitor concentration. The inhibitory behavior of **7**, as deduced from Figure 2, is strictly similar to that displayed by some reported bis-tetrahydroaminoacridine inhibitors of AChE. These compounds bind simultaneously at the catalytic and the peripheral sites of AChE and are characterized by a linear mixed type of enzyme inhibition.[19] Therefore, we concluded that **7** causes a mixed type of inhibition, i.e., inhibition of both the active site and a second distal site of the enzyme. Once verified that **7** may interact also with PAS of AChE, we verified whether there is a concomitant inhibition of Aβ aggregation induced by AChE through a thioflavin T-based fluorometric assay.[16,20] It turned out that TC and LA, i.e., the pharmacophoric moieties combined in **7**, were not able to inhibit at 100 μM the Aβ aggregation induced by AChE, whereas 100 μM **15** caused only a 25 ± 5% inhibition. In contrast, **7** was only 3-fold less potent than propidium, which is the most potent inhibitor of AChE-induced A^{β} aggregation so far available,[16] as revealed by an analysis of their IC_{50} values of 45.0 ± 14.6 and 12.6 ± 0.5 μM, respectively. Furthermore, **7** was significantly more potent than all the other AChE inhibitors ever tested,[16] including AP2238, an inhibitor purposely designed to bind the catalytic and the peripheral sites of AChE (35% of inhibition at 100 μM).[21]

Clearly, this finding, together with the results observed for **15**, TC, and LA separately, is relevant because an association of 100 μM TC and 100 μM LA or 100 μM **15** and 100 μM LA produced only a weak inhibition (15 ± 6% or 30 ± 7%, respectively) of AChE-induced Aβ aggregation, suggesting

that marked Aβ aggregation inhibition may be achieved only when the two prototypes are combined into the same structure, as in **7**.

The cytotoxicity effects of LA, **7**, and **15** were first determined by colorimetric MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay in human neuronal-like cells, SH-SY5Y, as described by Mosmann et al.[22] As reported in Figure 3, treatment of SH-SY5Y cells with LA and **7** (0.1-50 μM) did not show modified cell viability. By contrast, the treatment of SH-SY5Y cells with **15** (0.1-50 μM) produced a strong decrease of cell viability for 10 μM (88%) and 50 μM (99%). The intracellular antioxidant activity of LA, **7**, and **15** against formation of reactive oxygen species (ROS) in SH-SY5Y cells after treatment with *tert*-butyl hydroperoxide, a compound used to induce oxidative damage, was then assessed. A range of concentrations of tested compounds that did not affect cell viability (0.1-50 μM for LA and **7**; 0.1-5 μM for **15**) were used. As shown in Table 2, treatment of SH-SY5Y cells with LA showed a significant ($p < 0.01$) decrease of ROS formation only with the highest concentration used (50 μM), while the treatment with **7** produced a strong dose-dependent inhibitory effect on the ROS formation. Significant inhibitory effects by **7** with respect to basal values were reached for 5 μM ($p < 0.01$), 10 μM ($p < 0.001$), and 50 μM ($p < 0.001$). When treated with **15** (0.1-5 μM), the neuronal cells did not show any difference on ROS formation. Taken together, these results show that LA and **7** did not affect the neuronal viability while **15** exerted neurotoxic effects. In addition, LA and **7** (but not **15**) were able to protect neuronal cells against ROS formation evoked by oxidative stress, with **7** being the most active against ROS formation (64% inhibition at 50 μM).

In conclusion, the present investigation has shown that it is possible to obtain multipotent drugs for the treatment of AD: lipocrine (**7**) emerged in in vitro models as an effective candidate to be investigated in vivo for its multiple biological properties, namely, inhibition of AChE and BChE activities, inhibition of AChE-induced Aβ aggregation, and ability to protect cells against ROS.

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Table 1. Inhibition of AChE and BChE Activities by **15**, Tacrine (TC), Lipoic Acid (LA), and Hybrid Derivatives **1-8** (See Figure 1 for Structures)

			IC_{50} ± SEM (nM)[a]	
compd	*n*	R	AChE	BChE
1	2	H	97.0 ± 3.6	47.5 ± 1.8
2	3	H	6.96 ± 0.45	12.0 ± 0.6
3	4	H	35.2 ± 2.2	5.04 ± 0.32
4	5	H	38.4 ± 2.3	1.48 ± 0.35
5	6	H	30.1 ± 1.5	3.24 ± 0.29
6	7	H	32.7 ± 1.3	8.58 ± 0.57
7	3	Cl	0.253 ± 0.016	10.8 ± 2.5
8			1090 ± 180	329 ± 28
15			21.5 ± 0.8	2580 ± 60
TC			424 ± 21	45.8 ± 3.0
LA			>1000000	>1000000

[a] Human recombinant AChE and BChE from human serum were used. IC_{50} values represent the concentration of inhibitor required to decrease enzyme activity by 50% and are the mean of two independent measurements, each performed in triplicate. See Supporting Information for details.

Table 2. Effects of the Hybrid Compound **7** and the Two Parent Compounds LA and **15** on Intracellular ROS Formation in Neuronal Cells[a]

	intracellular ROS, %		
concn, μM	LA	**7**	**15**
0	86.00 ± 9.46	86.00 ± 9.46	86.00 ± 9.46
0.1	91.25 ± 2.99	88.75 ± 8.41	99.50 ± 3.54
0.5	90.25 ± 6.65	95.00 ± 5.20	99.67 ± 7.02
1	83.00 ± 7.58	77.00 ± 8.76	96.67 ± 8.02
5	79.50 ± 8.06	61.67 ± 9.02*	82.50 ± 7.78
10	74.33 ± 3.51	51.25 ± 9.21**	tox[b]

50	58.50 ± 9.19*	30.50 ± 9.04**	tox[b]

[a] The results are expressed as percentage increase of intracellular ROS evoked by exposure to *tert*-butyl hydroperoxide. Values are the mean ± SD of three independent experiments (treated vs untreated; (*) $p < 0.01$, (**) $p < 0.001$).[b] tox = cytotoxicity. See Supporting Information for details.

1. Synthesis and characterization of compounds 1-8

Compounds **1-8** were synthesized according to Scheme 1, coupling tetrahydroacridine intermediates with lipoic acid.

Melting points were taken in glass capillary tubes on a Büchi SMP-20 apparatus and are uncorrected. IR, electron impact (EI) mass, and direct infusion ESI-MS spectra were recorded on Perkin-Elmer 297, VG 7070E, and Waters ZQ 4000 apparatus, respectively. ^{1}H NMR, ^{13}C NMR, gHSQC and COSY experiments were recorded on Mercury 400 and Varian VXR 200 and 300 instruments. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), and spin multiplicities are given as s (singlet), d (doublet), dd (double doublet), t (triplet), or m (multiplet). Although the IR spectra data are not included (because of the lack of unusual features), they were obtained for all compounds reported and were consistent with the assigned structures. The elemental compositions of the compounds agreed to within ± 0.4% of the calculated value. When the elemental analysis is not included, crude compounds were used in the next step without further purification. Chromatographic separations were performed on silica gel columns by flash (Kieselgel 40, 0.040-0.063 mm; Merck) or gravity column (Kieselgel 60, 0.063-0.200 mm; Merck) chromatography. Compounds were named following IUPAC rules as applied by Beilstein-Institut AutoNom (version 2.1), a PC integrated software package for systematic names in organic chemistry.

(3-Aminomethyl-6-chloro-1,2,3,4-tetrahydroacridin-9-yl)amine (16). The synthesis of compound **16** was achieved by condensation of 2-amino-4-chlorobenzonitrile with 3-nitromethylcyclohexanone followed by reduction of the nitro group according to Rosini et al.,[1] and the isomeric conformation was assigned by means of ^{1}H NMR, ^{13}C NMR, gHSQC, and COSY experiments. Total yield 30%; mp 285-288 °C; ^{1}H NMR (400 MHz, CD_3OD) δ 7.91 (d, $J = 8.9$ Hz, 1H, C8-H), 7.58 (d, $J = 2.3$ Hz, 1H, C5-H), 7.19 (dd, $J = 9.0$, 2.3 Hz, 1H, C7-H), 2.86-2.94 (m, 1H, C4-H), 2.60-2.69 (m, 3H, -C*H*$_2$NH$_2$, C1-H), 2.19-2.25 (m, 2H, C1-H, C4-H), 2.04-2.13 (m, 1H, C2-

H), 1.75-1.83 (m, 1H, C3-H), 1.29-1.39 (m, 1H, C2-H); ^{13}C NMR (100 MHz, CD_3OD) δ 158.9, 150.3, 147.7, 135.2, 126.1 (C5), 124.6 (C7), 124.1 (C8), 116.3, 110.4, 48.1 (-*C*H$_2$NH$_2$), 38.3 (C4), 37.9 (C3), 27.3 (C2), 24.2 (C1); EI MS *m/z* 261 (M+).

General procedure for the synthesis of compounds 1-8.

A solution of the appropriate tetrahydroacridinamine (1 eq) and lipoic acid (1.5 eq) in dry DMF (5 mL), under N_2, was cooled to 0 °C and then treated with 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (1.2 eq): the mixture was stirred at 0 °C for further 15 min and then at rt for 2 h in the dark. Solvent was then removed under vacuum, avoiding heating up the reaction mixture, affording an oily residue that was purified by gravity column.

5-([1,2]Dithiolan-3-yl)-*N*-{[2-(1,2,3,4-tetrahydroacridin-9-yl)amino]ethyl}pentanamide (1). It was synthesized from *N*1-(1,2,3,4-tetrahydroacridin-9-yl)ethane-1,2-diamine (**9**)$_2$ (140 mg). Elution with petroleum ether/CH_2Cl_2/MeOH/aqueous 30% ammonia (6:3:1:0.055) afforded **1** as a foam solid: 35% yield; ^{1}H NMR (300 MHz, CD_3OD) δ 8.12 (d, J = 8.8 Hz, 1H), 7.78 (d, J = 8.8 Hz, 1H), 7.58, (t, J = 8.2 Hz, 1H), 7.39 (t, J = 8.2 Hz, 1H), 3.70 (t, J = 6.3 Hz, 2H), 3.28-3.39 (m, 3H), 2.93-3.15 (m, 4H), 2.71-2.79 (m, 2H), 2.26-2.40 (m, 1H), 2.15 (t, J = 8.6 Hz, 2H), 1.64-1.93 (m, 5H), 1.30-1.61 (m, 6H); MS (ESI+) *m/z* 430 (M+H)+. Calcd. for $C_{23}H_{31}N_3OS_2$: C, 64.30; H, 7.27; N, 9.78; found C, 64.41; H, 7.28; N, 9.75.

5-([1,2]Dithiolan-3-yl)-*N*-{[3-(1,2,3,4-tetrahydroacridin-9-yl)amino]propyl}pentanamide (2). It was synthesized from *N*1-(1,2,3,4-tetrahydroacridin-9-yl)propane-1,3-diamine (**10**) (100 mg, obtained from 9-chloro-1,2,3,4-tetrahydroacridine and propane-1,3-diamine following the procedure described in Carlier et al.,[3] and purified by flash chromatography with a step gradient system of CH_2Cl_2/MeOH/ aqueous 30% ammonia (9.5:0.5:0.0 to 7:3:0.1): 65% yield, ^{1}H NMR (200 MHz, CD_3OD) δ 8.08 (d, J = 8.8 Hz, 1H), 7.78 (d, J = 8.7 Hz, 1H), 7.53, (t, J = 8.3 Hz, 1H), 7.32 (t, J = 8.3 Hz, 1H), 3.54 (t, J = 6.7 Hz, 2H), 2.87-2.98 (m, 2H), 2.65 (t, J = 7.5 Hz, 4H), 1.64-1.93 (m, 6H)). Elution with petroleum ether/CH_2Cl_2/MeOH/aqueous 30% ammonia (5:4:1:0.05) afforded **2** as a foam solid: 35% yield; ^{1}H NMR (200 MHz, CD_3OD) δ 8.15 (d, J = 8.8 Hz, 1H), 7.78 (d, J =

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8.8 Hz, 1H), 7.56-7.64 (m, 1H), 7.37-7.44 (m, 1H), 3.69 (t, J = 6.6 Hz, 2H), 3.40-3.52 (m, 1H), 3.23-3.36 (t, J = 6.6 Hz, 2H), 2.92-3.18 (m, 4H), 2.74-2.83 (m, 2H), 2.28-2.43 (m, 1H), 2.19 (t, J = 7.1 Hz, 2H), 1.73-1.95 (m, 7H), 1.22-1.68 (m, 6H);); MS (ESI+) *m/z* 444 (M+H)+. Calcd. for $C_{24}H_{33}N_3OS_2$: C, 64.97; H, 7.50; N, 9.47; found C, 65.18; H, 7.52; N, 9.44.

5-([1,2]Dithiolan-3-yl)-*N*-{[4-(1,2,3,4-tetrahydroacridin-9-yl)amino]butyl}pentanamide (3). It was synthesized from *N*1-(1,2,3,4-tetrahydroacridin-9-yl)butane-1,4-diamine (**11**)$_3$ (290 mg). Elution with petroleum ether/CH_2Cl_2/MeOH/aqueous 30% ammonia (6:3:1:0.06) afforded **3** as a foam solid: 38% yield; ^{1}H NMR (200 MHz, CD_3OD) δ 8.12 (d, J = 8.6 Hz, 1H), 7.78 (d, J = 8.6 Hz, 1H), 7.52-7.62 (m, 1H), 7.32-7.43 (m, 1H), 3.41-3.60 (m, 3H), 2.90-3.21 (m, 6H), 2.68-2.77 (m, 2H), 2.31-2.46 (m, 1H), 2.17 (t, J = 6.9 Hz, 2H), 1.38-1.95 (m, 15H); MS (ESI+) *m/z* 458 (M+H)+.

Calcd. for $C_{25}H_{35}N_3OS_2$: C, 65.60; H, 7.71; N, 9.18; found C, 65.67; H, 7.69; N, 9.15.

5-([1,2]Dithiolan-3-yl)-*N*-{[5-(1,2,3,4-tetrahydroacridin-9-yl)amino]pentyl}pentanamide (4). It was synthesized from *N*1-(1,2,3,4-tetrahydroacridin-9-yl)pentane-1,5-diamine (**12**)[3] (480 mg). Elution with petroleum ether/CH_2Cl_2/MeOH/aqueous 30% ammonia (6:3:1:0.055) afforded **4** as a foam solid: 40% yield; ^{1}H NMR (200 MHz, CD_3OD) δ 8.09 (d, J = 8.6 Hz, 1H), 7.78 (d, J = 8.6 Hz, 1H), 7.52-7.60 (m, 1H), 7.33-7.41 (m, 1H), 3.40-3.57 (m, 3H), 2.87-3.18 (m, 6H), 2.63-2.75 (m, 2H), 2.25-2.43 (m, 1H), 2.17 (t, J = 6.8 Hz, 2H), 1.35-1.95 (m, 17H); MS (ESI+) *m/z* 472 $(M+H)^+$. Calcd. for $C_{26}H_{37}N_3OS_2$: C, 66.20; H, 7.91; N, 8.91; found C, 66.41; H, 7.89; N, 8.88.

5-([1,2]Dithiolan-3-y)-N-{[6-(1,2,3,4-tetrahydroacridin-9-yl)amino]hexyl}pentanamide (5). It was synthesized from *N*1-(1,2,3,4-tetrahydroacridin-9-yl)hexane-1,6-diamine (**13**)[3] (370 mg). Elution with petroleum ether/CH_2Cl_2/MeOH/aqueous 30% ammonia (6:3:1:0.05) afforded **5** as a foam solid: 30% yield; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.83 (apparent t, J = 9.3 Hz, 2H), 7.47-7.56 (m, 1H), 7.28-7.37 (m, 1H), 5.89 (t, J = 3.2 Hz, 1H, exchangeable with D_2O), 4.15 (br s, 2H, exchangeable with D_2O), 3.40-3.57 (m, 3H), 3.01-3.23 (m, 6H), 2.60-2.75 (m, 2H), 2.31-2.48 (m, 1H), 2.15 (t, J = 7.3 Hz, 2H), 1.35-1.96 (m, 19H); MS (ESI+) *m/z* 486 $(M+H)^+$. Calcd. for $C_{27}H_{39}N_3OS_2$: C, 66.76; H, 8.09; N, 8.65; C, 66.87; H, 8.12; N, 8.62.

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5-([1,2]Dithiolan-3-y)-*N*-{[7-(1,2,3,4-tetrahydroacridin-9-yl)amino]heptyl}pentanamide (6). It was synthesized from *N*1-(1,2,3,4-tetrahydroacridin-9-yl)heptane-1,7-diamine (**14**)[3] (220 mg). Elution with petroleum ether/CH_2Cl_2/MeOH/aqueous 30% ammonia (6:3:1:0.05) afforded **6** as a foam solid: 35% yield; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.92 (apparent t, J = 9.4 Hz, 2H), 7.51-7.61 (m, 1H), 7.30-7.41 (m, 1H), 5.57 (t, J = 3.2 Hz, 1H, exchangeable with D_2O), 3.40-3.61 (m, 3H), 3.01-3.24 (m, 6H), 2.64-2.73 (m, 2H), 2.38-2.54 (m, 1H), 2.18 (t, J = 7.3 Hz, 2H), 1.25-1.98 (m, 21H); MS (ESI+) *m/z* 500 $(M+H)^+$. Calcd. for $C_{28}H_{41}N_3OS_2$: C, 67.29; H, 8.27; N, 8.41; C, 67.43; H, 8.30; N, 8.39.

5-([1,2]Dithiolan-3-yl)-*N*-[3-(6-chloro-1,2,3,4-tetrahydro-acridin-9-yl)amino]propyl}pentanamide (7). It was synthesized from *N*1-(6-chloro-1,2,3,4-tetrahydroacridin-9-yl)propane-1,3-diamine (**15**) (180 mg, obtained from 6,9-dichloro-1,2,3,4-tetrahydroacridine and propane-1,3-diamine following the procedure described in Carlier et al.,[3] and purified by flash chromatography with a step gradient system of CH_2Cl_2/MeOH/ aqueous 30% ammonia (9.5:0.5:0.0 to 8:2:0.03): 70% yield, ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.93 (d, J = 9.1 Hz, 1H), 7.86 (d, J = 2.4 Hz, 1H), 7.22 (dd, J = 9.0, 2.3 Hz, 1H), 3.62 (t, J = 6.8 Hz, 2H), 2.88-3.05 (m, 4H), 2.60-2.68 (m, 2H), 1.71-1.95 (m, 6H)). Elution with petroleum ether/CH_2Cl_2/EtOH/aqueous 30% ammonia (7:2:1:0.03) afforded **7** as a foam solid: 35% yield; ^{1}H NMR (200 MHz, CD_3OD) δ

8.08 (d, J = 8.9 Hz, 1H), 7.72 (d, J = 2.1 Hz, 1H), 7.28 (dd, J = 8.9, 2.1 Hz, 1H), 3.42-3.58 (m, 3H), 3.27 (t, J = 6.5 Hz, 2H), 2.89-3.17 (m, 4H), 2.65-2.77 (m, 2H), 2.27-2.43 (m, 1H), 2.19 (t, J = 7.2 Hz, 2H), 1.73-1.91 (m, 7H), 1.31-1.65 (m, 6H); MS (ESI+) *m/z* 478 (M+H)+. Calcd. for $C_{24}H_{32}ClN_3OS_2$: C, 60.29; H, 6.75; N, 8.79; found C, 60.45; H, 6.74; N, 8.77.

***N*-[(9-Amino-6-chloro-1,2,3,4-tetrahydroacridin-3-yl)methyl]-5-[1,2]dithiolan-3-yl)pentanamide (8).** It was synthesized from **1 6** (150 mg). Elution with CH_2Cl_2/toluene/EtOH/aqueous 30% ammonia (5:3:2:0.02) afforded **8** as a foam solid: 30% yield; ^{1}H NMR (200 MHz, CD_3OD) δ 8.09 (d, J = 8.9 Hz, 1H), 7.72 (d, J = 2.2 Hz, 1H), 7.36 (dd, J = 9.2, 2.2 Hz, 1H), 3.50-3.62 (m, 2H), 2.96-3.21 (m, 4H), 2.70-2.83 (m, 1H), 2.38-2.69 (m, 3H), 2.28 (t,

7.0 Hz, 2H), 2.05-2.21 (m, 2H), 1.79-1.95 (m, 1H), 1.23-1.78 (m, 7H); EI MS *m/z* 449 (M+). Calcd. for $C_{22}H_{28}ClN_3OS_2$: C, 58.71; H, 6.27; N, 9.34; found C, 58.91; H, 6.26; N, 9.31.

2. Biology

Inhibition of AChE and BChE. The method of Ellman et al. was followed.[4] Five different concentrations of each compound were used in order to obtain inhibition of AChE or BChE activity comprised between 20-80%. The assay solution consisted of a 0.1 M phosphate buffer pH 8.0, with the addition of 340 μM 5,5'-dithio-bis(2-nitrobenzoic acid), 0.02 unit/mL of human recombinant AChE or human serum BChE (Sigma Chemical), and 550 μM of substrate (acetylthiocholine iodide or butyrylthiocholine iodide). Test compounds were added to the assay solution and preincubated at 37 °C with the enzyme for 20 min followed by the addition of substrate. Assays were done with a blank containing all components except AChE or BChE in order to account for non-enzymatic reaction. The reaction rates were compared and the percent inhibition due to the presence of test compounds was calculated. Each concentration was analyzed in triplicate, and IC_{50} values were determined graphically from log concentration–inhibition curves.

Determination of Steady State Inhibition Constant. To obtain estimates of the competitive inhibition constant K_i, reciprocal plots of 1/V versus 1/[S] were constructed at relatively low concentration of substrate (below 0.5 mM). The plots were assessed by a weighted least square analysis that assumed the variance of V to be a constant percentage of V for the entire data set. Slopes of these reciprocal plots were then plotted against the concentration of 7 (range 0 – 0.344 nM) in a weighted analysis and K_i was determined as the ratio of the replot intercept to the replot slope.

Reciprocal plots involving TC (not shown) or 7 inhibition show both increasing slopes (decreased V_{max} at increasing inhibitor's concentrations) and increasing intercepts (higher K_m) with higher inhibitor concentration. This pattern indicates mixed inhibition, arising from significant

inhibitor interaction with both the free enzyme and the acetylated enzyme. Replots of the slope

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versus the concentration of 7 or TC gives estimate of competitive inhibition constant, $K_i = 0.155 \pm 0.046$ nM or $K_i = 0.151 \pm 0.016$ μM, respectively.

So the pattern in the graphical representation shows 7 able to bind to the peripheral anionic site as well as the active site of AChE.

Inhibition of AChE-induced A β aggregation. Aliquots of 2 μL A β peptide, lyophilized from 2 mg mL^{-1} 1,1,1,3,3,3-hexafluoro-2-propanol solution and dissolved in DMSO, were incubated for 24 h at room temperature in 0.215 M sodium phosphate buffer (pH 8.0) at a final concentration of 230 μM. For co-incubation experiments aliquots (16 μL) of AChE (final concentration 2.30 μM, Aβ/AChE molar ratio 100:1) and AChE in the presence of 2 μL of the tested inhibitor in 0.215 M sodium phosphate buffer pH 8.0 solution (final inhibitor concentration 100 μM) were added. Blanks containing A β, AChE, and A β plus inhibitors at various concentrations, in 0.215 M sodium phosphate buffer (pH 8.0) were prepared. The final volume of each vial was 20 μL. Each assay was run in duplicate. To quantify amyloid fibril formation, the thioflavin T (ThT) fluorescence method was then applied.[5] After dilution with glycine-NaOH buffer (pH 8.5), containing 1.5 mM ThT, the fluorescence intensities due to β-sheet conformation was monitored for 300 s at $\lambda_{em} = 490$ nm ($\lambda_{ex} = 446$ nm). The percent inhibition of the AChE induced aggregation due to the presence of the test compound was calculated by the following expression: $100-(IF_i/IF_o \times 100)$ where IF_i and IF_o are the fluorescence intensities obtained for Aβ plus AChE in the presence and in the absence of inhibitor, respectively, minus the fluorescent intensities due to the respective blanks.

Cell cultures. Human neuronal-like cells, SH-SY5Y, were routinely grown at 37°C in a humidified incubator with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin.

Determination of cytotoxicity. The cytotoxicity was evaluated with the colorimetric MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide] assay, as described by Mosmann et al.[6] Briefly, SH-SY5Y cells were seeded in 96-well microtiter plates at 2×10^5 cells/well. After

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24 h of incubation at 37°C in 5% CO_2, the growth medium was removed and media containing compounds (0.1-50 μM) were added to the cells. After 24 h of incubation, the cells were washed with phosphate buffered saline (PBS) and then incubated with MTT (5 mg/ml) in PBS for 4 h. After removal of MTT and further washing, the formazan crystals were dissolved with isopropanol. The amount of formazan was measured (405 nm) with a spectrophotometer (TECAN®, Spectra model Classic, Salzburg, Austria). The cell viability was expressed as percentage of control cells and

calculated by the formula $F_t/F_{nt} \times 100$, where F_t = absorbance of treated neurones and F_{nt} = absorbance of untreated neurones.

Determination of antioxidant activity. The antioxidant activity of compounds was evaluated by measuring the formation of intracellular reactive oxygen species (ROS) evoked by exposure of SH-SY5Y cells to *tert*-butyl hydroperoxide (*t*-BuOOH), a compound used to induce oxidative stress. Formation of intracellular ROS was determined using a fluorescent probe, DCFH-DA, as described by Wang H. et al.[7] Briefly, SH-SY5Y cells were seeded in 96-well microtiter plates at 2×10^5 cells/well. After 24 h of incubation at 37°C in 5% CO_2, the growth medium was removed and media containing compounds (0.1-50 μM) were added to the cells. After 24 h of incubation, the cells were washed with PBS and then incubated with 5μM of DCFH-DA in PBS at 37°C in 5% CO_2 for 30 min. After removal of DCFH-DA and further washing, the cells were incubated with 0.1 mM *t*-BuOOH in PBS for 30 min. At the end of incubation, the fluorescence of the cells from each well was measured ($\lambda_{excitation}$ = 485 nm, $\lambda_{emission}$ = 535 nm) with a spectrofluorometer (Wallac Victor□ Multilabel Counter, Perkin Elmer Inc., Boston, MA). The results were expressed as percentage increase of intracellular ROS evoked by exposure to *t*-BuOOH and calculated by the formula $[(F_t - F_{nt}) / F_{nt} \times 100]$, where F_t = fluorescence of treated neurones and F_{nt} = fluorescence of untreated neurones.

Statistical analysis. Data are reported as mean ± SD of at least 3 independent experiments. Statistical analysis was performed using ANOVA (Scheffe post hoc test was used) and the

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differences were considered significant at $p < 0.05$. Analyses were performed using STATISTICA 4.5 software on a Windows platform.

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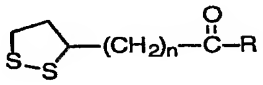
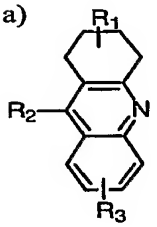
The general structure of lipoic acid derivatives can be schematized as such:



Where n = 1-4;
Examples where R =:

a)

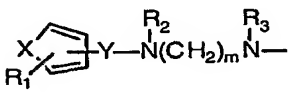


- R_1 = H, alkylamine, nitroalkyl, halogen, hydroxy
- R_2 = alkanediamine, amino
- R_3 = halogen, H, methoxy

The examples are structures (**1-8**) reported in the enclosed manuscript;

An other example is an inferior homologous of **8**:
RM11: n = 1, R_1 = 3-ylmethylamine, $R_2 = NH_2$, R_3 = 6-chloro;

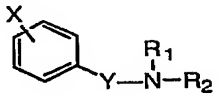
b)



Example:

MR106: n = 4; X = CH=CH, R_1 = 2-MeO, m = 6; R_2 = Et, R_3 = H.

c)



R_1 = alkyl, R_2 = alkyl, alkylamine

Y = alkyl

X = OH, O-carbamoyl, O-

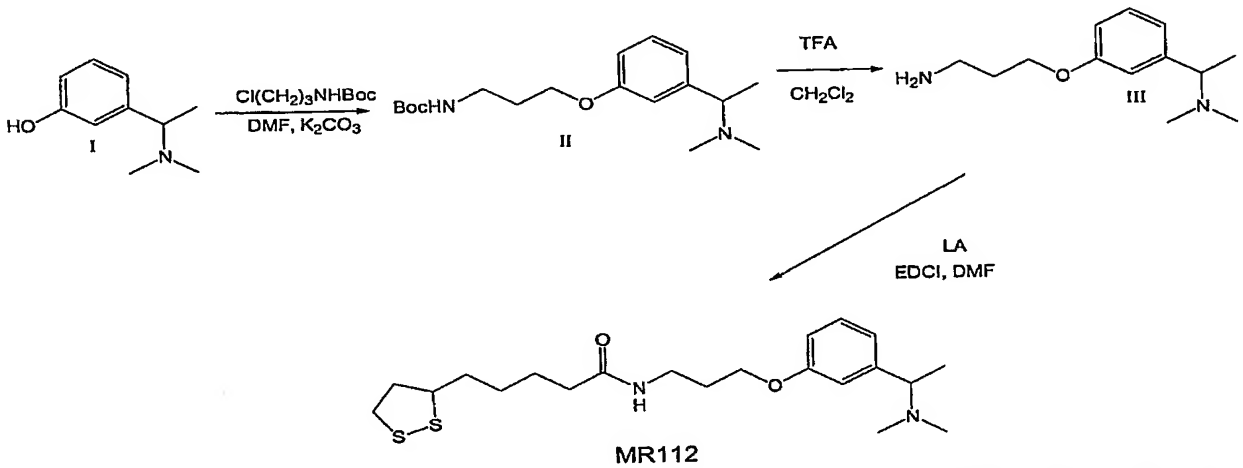
An example is **MR112**: Y = MeCH, R_1 = R_2 = Me, X = O-.

Synthesis of examples:

***N*-(9-Amino-6-chloro-1,2,3,4-tetrahydro-acridin-3-yl-methyl)-2-[1,2]ditiolan-3-il-acetamide (RM11).** A solution of 3-aminomethyl-6-chloro-1,2,3,4-tetrahydro-acridin-9-ylamine[i] (140 mg, 0.53 mmol) and [1,2]Dithiolan-3-yl-acetic acid[ii] (90 mg, 0.55 mmol) in dry DMF (5 mL), under N_2, was cooled to 0 °C and then added of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDCI) (113 mg, 0.59 mmol). The mixture was stirred at 0 °C for further 15 min and then at rt for 2 h in the dark. Solvent was then removed under vacuum, avoiding heating up the reaction mixture, affording a residue that was purified by gravity column. Elution with petroleum ether/CH_2Cl_2/MeOH/aqueous 30% ammonia (5:4:1:0.1) afforded **RM11** as a foam solid: 40% yield; ^{1}H NMR (200 MHz, CD_3OD) δ 8.03 (d, *J* = 9.2 Hz, 1H), 7.67 (d, *J* = 2.2 Hz, 1H), 7.30 (dd, *J* = 8.8, 2.2 Hz, 1H), 4.05 (m, 1H), 2.95-3.34 (m, 5H), 2.46-2.59 (m, 6H), 1.99-2.20 (m, 3H), 1.48-1.52 (m, 1H). Anal. $C_{19}H_{22}ClN_3OS_2$: C, 55.93; H, 5.44; N, 10.30. Found: C, 56.01; H, 5.45, N, 10.11.

5-[1,2]Dithiolan-3-yl-pentanoic acid {6-[Ethyl-(2-methoxy-benzyl)-amino]-esyl}-amide (MR106) was synthesized from *N*^{1}-ethyl-*N*1-(2-methoxy-benzyl)-hexano-1,6-diamine[iii] (300 mg, 1.13 mmol) and lipoic acid (350 mg, 1.70 mmol) according to the procedure described for **RM11**, and purified by gravity column. Elution with a gradient of mobile phase petroleum ether/toluene/CH_2Cl_2/EtOH/aqueous 30% ammonia (from 7:2:1:1:0.05 to 7:1:1:1:0.05) afforded **MR106** as solid foam: yield 43%, ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.42-7.48 (m, 1H); 7.18-7.26 (m, 1H), 6.85-6.99 (m, 2H), 5.43 (br t, 1H, exchangeable with D_2O), 3.84 (s, 3H), 3.53-3.64 (m, 1H + s, 2H), 3.08-3.27 (m, 4H), 2.43-2.57 (m, 5H), 2.17 (t, *J* = 7.4 Hz, 2H), 1.82-2.00 (m, 1H), 1.29-1.73 (m, 14H), 1.07 (t, *J* = 7.0 Hz, 3H); MS (ESI$^+$) *m/z* 453 $(M+H)^+$. Calculated Anal. for $C_{24}H_{40}N_2O_2S_2$: C, 63.67; H, 8.91; N, 6.19. Found: C, 63.79; H, 8.93, N, 6.17.

{2-[3-(1-dimethylamino-ethyl)-phenoxy]-ethyl}-carbamic acid Tert-butyl ester (II). A solution of $\mathbf{I}^{iv}$ (350 mg, 2.17 mmol), (3-chloro-propyl)-carbamic acid tert-butylic ester (420 mg, 2.17 mmol) and K_2CO_3 (300 mg, 2.17 mmol) in DMF (10 ml) was stirred under reflux for 24 h . Solvent evaporation afforded a residue that was purified by gravity column. Elution with $CHCl_3$/MeOH/ aqueous 30% ammonia (9:1:0.02) afforded **II** as an oil: yield 65%, ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.20 (t, J = 8.0 Hz, 1H); 6.79-6.89 (m, 3H), 4.92 (br s, 1H, exchangeable with D_2O), 4.02 (t, J = 6.4 Hz, 2H); 3.20-3.33 (m, 3H), 2.20 (s, 6H), 1.93-1.99 (m, 2H), 1.44 (s, 9H), 1.35 (d, J = 6.6 Hz, 3H).



MR112

{2-[3-(1-dimethylamino-ethyl)-phenoxy]-ethyl}-carbamic acid tert-butyl ester (II). A solution of $\mathbf{I}^{iv}$ (350 mg, 2.17 mmol), (3-chloro-propyl)-carbamic acid tert-butylic ester (420 mg, 2.17 mmol) and K_2CO_3 (300 mg, 2.17 mmol) in DMF (10 ml) was stirred under reflux for 24 h. Solvent evaporation afforded a residue that was purified by gravity column. Elution with $CHCl_3$/MeOH/ aqueous 30% ammonia (9:1:0.02) afforded **II** as an oil: yield 65%, ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.20 (t, J = 8.0 Hz, 1H); 6.79-6.89 (m, 3H), 4.92 (br s, 1H, exchangeable with D_2O), 4.02 (t, J = 6.4 Hz, 2H); 3.20-3.33 (m, 3H), 2.20 (s, 6H), 1.93-1.99 (m, 2H), 1.44 (s, 9H), 1.35 (d, J = 6.6 Hz, 3H).

3-[3-(1-Dimetylamino-ethyl)-phenoxy]-propilamine (III). A solution of **II** (200 mg, 0.62 mmol) in CH_2Cl_2 (5 mL) is added with TFA (1.5 mL) and stirred at room temperature for 2 h. The reaction mixture was evaporated under vacuum, the afforded residue was dissolved in water, reso basico by adding NaOH 2 N and then extracted with $CHCl_3$ (3 x 20 mL). The evaporation of the dried solvent afforded **III** as an oil; quantitative yield, ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.20 (t, J = 8.0

Hz, 1H); 6.72-6.88 (m, 3H), 4.04 (t, J = 6.2 Hz, 2H); 3.12-3.22 (m, 1H), 2.91 (t, J = 6.6 Hz, 2H), 2.19 (s, 6H), 1.88-1.95 (m, 2H), 1.43 (br s, 2H, exchangeable with D_2O), 1.34 (d, J = 6.6 Hz, 3H).

5-[1,2]Dithiolan-3-yl-pentanoic acid {3-[3-(1-Dimethylamino-ethyl)-phenoxy]-propyl}-amide (MR112) was synthesized from **III** (150 mg, 0.67 mmol) and lipoic acid (210 mg, 1.02 mmol) according to the procedure described for **RM11**, and purified by gravity column. Elution with petroleum ether/toluene/CH_2Cl_2/MeOH/ aqueous 30% ammonia (6:1:1.5:1.5:0.01) afforded **MR112** as a solid foam ceroso; yield 30%, ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.27 (t, J = 8.2 Hz, 1H); 6.78-6.98 (m, 3H), 5.99 (br t, 1H); 4.09 (t, J = 6.0 Hz, 2H); 3.21-3.62 (m, 5H), 3.05-3.19 (m, 3H), 2.40-2.53 (m, 1H), 2.32 (s, 6H), 2.22 (t, J = 7.2 Hz, 2H); 1.81-2.0 (m, 3H), 1.65-1.73 (m, 4H), 1.47 (d, J = 6.6 Hz, 3H) ; MS (ESI^+) *m/z* 411 $(M+H)^+$. Calculated Anal. for $C_{21}H_{34}N_2O_2S_2$: C, 61.42; H, 8.35; N, 6.82. Found: C, 61.62; H, 8.36, N, 6.80.

[i] Present document

[ii] Chen, Yaun-Shek and Lawton, Richard G. An efficient synthetic route to 2-(1,2-dithiolan-3-yl)acetic acid. Trisnorlipoic acid and amide derivatives. *Tetrahedron Letters* **1997**, *38*, 5785-5788.

[iii] Patent UNIBO-LLG.

[iv] **I** was synthesized according to the procedure described for the corresponding (R,S)-3-[[1-di-($^2H_3$) methylamino]ethyl]phenol in: Ciszewska, Grazyna; Pfefferkorn, Heidi; Tang, Y.S.; Jones, Lawrence; Tarapata, Richard; Sunay, Ustun B. Synthesis of tritium, deuterium, and carbon-14 labeled (s)-n-ethyl-n-methyl-3-[1-(dimethylamino)ethyl]carbamic acid, phenyl ester, (l)-2,3-dihydroxybutanedioic acid salt (SDZ ENA 713 hta), an investigational drug for the treatment of Alzheimer's disease. *Journal of Labelled Compounds & Radiopharmaceuticals* **1997**, *39*, 651-668.

Figure and Scheme Legends

Figure 1. Design strategy for compounds **1-8**.

Figure 2. Steady–state inhibition of AChE hydrolysis of acetylthiocholine (ACTh) by **7**. Linewaver-Burk reciprocal plots of initial velocity and substrate concentrations are reported. Reciprocal plots of initial velocity in the absence of inhibitor gave an estimate of k_{app} for acetylthiocholine of 170 ± 15 μM (four experiments). Lines were derived from a weighted least-squares analysis of the data points.

Figure 3. Effects of compounds on cell viability in neuronal cells. The cell viability was determined by the MTT assay (as described in the experimental section), after 24 h of incubation with various concentrations of LA (solid circle), **7** (open circle) and **15** (open triangle). The results were expressed as percentage of control cells. Values are reported as mean ± SD of three independent experiments.

Scheme 1. Synthesis of compounds **1-8**.

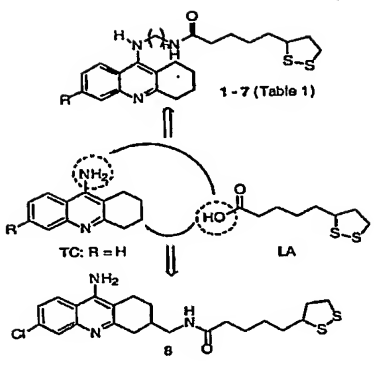


Figure 1

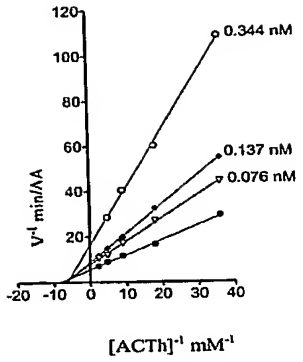


Figure 2

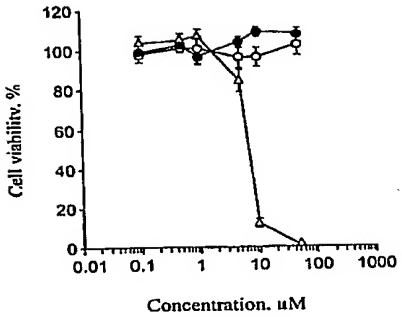
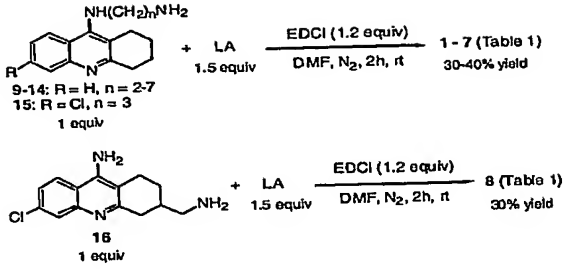


Figure 3



Scheme 1